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# PRODUCTION METHOD OF GLYCOPROTEIN

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[There are no amendments in this translation]

Abstract

Constitution

A method that modifies the type or the molecular weight of the sugar chain in a glycoprotein that is produced by changing the sugar composition or the sugar concentration in the medium at the production of the glycoprotein by culturing an animal cell in a medium.

Effect

Sugar chains in glycoproteins can be modified by changing the sugar composition and the sugar concentration in the medium, and glycoproteins with different characteristics can be optionally obtained by an easy method.

Claim

A glycoprotein production characterized in that the type or the molecular weight of the sugar chain that binds with protein is modified by changing the sugar composition and/or sugar concentration in the medium at the production of glycoprotein by culturing an animal cell in a medium.

Detailed explanation of the invention

[0001]

Industrial application field

The present invention concerns a glycoprotein production method with modified characteristics by modifying the type or the molecular weight of the sugar chain that is bound to proteins by controlling the cell culture conditions, in particular the medium conditions at the production of glycoprotein by culturing an animal cell in a medium.

[0002]

Prior art

Physiologically active proteins such as lymphokines, cytokines, hormones, and immunoglobulins, for example, that play significant in vivo roles are mostly glycoproteins that are bound to sugar chains. The polypeptide part of these glycoproteins can be mass-produced by genetic recombination methods if that gene is cloned. However, sugar chains do not bind in prokaryotic cells like *Escherichia coli*, for example, in the production of glycoproteins, therefore, the production generally uses mammalian cells. Although the role the sugar chain in the glycoprotein plays has not been completely clarified, from the study of the proteins that are produced from *Escherichia coli* in which sugar chains do not bind, it is known that a protein that has deleted sugar chains when compared to the original glycoprotein has a significantly reduced in vivo half-life (Murakami et al., Agricultural Chemistry 62:1498-1510, 1988). It also has been clarified that the sugar chain that binds with the Fc part in an antibody is mandatory in the manifestation of the Fc activity, and it is also mandatory in the manifestation of in vivo activity with erythropoietin, which is a hematinic hormone. It has also been clarified that sugar chains participate in the antigentic specificity of the antibody. As a result of the obtention of a hybridoma that produces a monoclonal antibody that has sugar chains on the L chain of the antibody and a detailed examination of its antigen recognition, Murakami et al. have confirmed the significant involvement of the presence of the L chain bound sugar chain to antigen recognition (Murakami H., et al., Animal Cell Technology; Basic & Applied Aspects, pp. 547-551, Kluver Academic Publishers, 1992). In this way, it has been clarified that the existence of the bound sugar chain plays a significant function in the manifestation of the activity of the physiologically active glycoprotein.

[0003]

It is thought that the peptide part of a sugar chain is synthesized in the cell nucleus [sic; cytoplasm] in an eukaryote, and sugar chain is added afterwards in the Golgi apparatus. Sugars that constitute sugar chains consist of 11 monosaccharides including glucose, galactose, mannose, hexoses such as N-acetylglucosamine, for example, and pentoses such as L-arabinose, for example. Nearly infinite sugar chains can be constructed by combining these sugar types. Binding of sugar chains changes due to the glycosyltransferases possessed by the cell. In a cell that has a genetic abnormality, like a cancerous cell for example, the manifestation of the glycosyltransferases goes wrong, and sugar chains that are different from those of normal cells are synthesized. It has been thought in the past that sugar chains that are characteristic to that cell line bind in binding of sugar chains. It is also well known that a protein that does not bind sugar chains can be obtained by culture with about 10 μg/mL tsunicamycin, which is a glycosylation

inhibitor in cells, added to prevent for sugar chains from binding. In binding of sugar chains by N-glycoside binding, N-acetyl glucosamine binds with asparagine (Asn, amino acids will be indicated by 3 letters below). In this case, sugar chains bind with Asn of the amino acid sequence that has the sequence of Asn-X-Ser (or Thr) (X indicates an optional amino acid). In the past, binding of sugar chains has been changed by changing the amino acid that participates in binding of this N-glycoside sugar chain at a genetic level, or sugar chains have been changed by changing a host cell by genetic recombination. The example of the production of erythropoietin can be named as a representative example of the latter. Erythropoietin is a glycoprotein with a molecular weight of about 34000, and this protein consists of 166 amino acids. It is known that its sugar chain structure will be different when a gene that codes for this amino acid sequence is introduced into CHO and $\phi$2 cells and expressed. This type of method for obtaining glycoproteins with different sugar chain structures by changing the host cell is known. A method that processes a glycoprotein using an enzyme like glucanase for partially cleaving the sugar chain has also been attempted, but it is not much suited for mass production. A method that modifies the sugar chain of a glycoprotein and obtains a glycoprotein that has a sugar chain with a new function is a technique that has been newly developed as in sugar chain engineering, However, the intended sugar chains have not yet been freely obtained under the present circumstances.

[0004]

Problem to be solved by the invention

In the process of the advancement of the study of the sugar chain structure of glycoproteins, the inventors of the present invention have discovered a phenomenon that is completely different from the production of glycoproteins that has been reported in the past. It has been reported that the sugar chain in a glycoprotein is characteristic to a cell like the host cell that manifests the glycoprotein as described above, and it has been thought that the sugar chain does not change unless there is a genetic mutation like carcinogenesis, for example. However, the inventors of this invention, when examining antibody-producing hybridoma cultures, have discovered that sugar chains that have been considered characteristic to cells change according to the cell culture conditions. This invention is based on such knowledge, and its task is to offer a glycoprotein production method that modifies the type or the molecular weight of the binding sugar chain by changing the sugar composition and/or the sugar concentration in the medium when culturing an animal cell for the production of a glycoprotein. It becomes possible to obtain materials with different activities and stabilities in the glycoprotein obtained by modifying the type or the molecular weight of the sugar chains.

[0005]

Means to solve the problem

In the conventional animal cell culture, the principle was to select the medium composition most suitable for the growth of cells and to change the culture conditions. Accordingly, the biggest challenge in the cell culturing was monitoring the various types of components that were consumed by the cell and supplying the decreased amounts of the respective components to maintain optimal conditions. The characteristic of this invention is culturing an animal cell that produces a glycoprotein under the different sugar concentration or sugar composition conditions that are considered optimal for cell growth. More precisely, this invention is a glycoprotein manufacturing method with the characteristic that the type and the molecular weight of the sugar chain that binds with a protein are modified by changing the sugar composition/or the sugar concentration in the medium at the production of glycoprotein by culturing an animal cell in a medium. Among animal cells, the growth conditions of mammalian cells are well known, and they are suitable for the implementation of this invention. As such mammalian animal cells, antibody-producing hybridomas, CHO, and C127 cells, for example, can be named. Glucose is generally used as the sugar in the culture solution for culturing an animal cell, however, fructose is rarely added with the purpose of inhibiting the formation of lactic acid in the culture solution. In this invention, when using glucose as the sugar, that the concentration is changed within a range of 0-40 mM, or the sugar in the culture solution is replaced by sugar that is present in the structure of the sugar chain of a glycoprotein, including fructose, mannose, galactose, N-acetylglucosamine, ribose, fucose, and N-acetylgalactosamine, for example, instead of glucose, and sugar chains can be replaced. The intended effect can also be obtained by adding a polysaccharide that contains sugar that makes up sugar chain like chitosan, dextran sulfate, and alginic acid, for example, into the medium. This is a totally new method as a method for changing sugar chains in glycoproteins.

[0006]

A cell that produces a target glycoprotein, particularly a cell line that is transformed by genetic recombination, is cultured by selecting a medium composition suitable for cell growth. For changing the binding state of the sugar chain, it is often desirable to culture at a sugar concentration lower than an optimal sugar concentration. A serum-free medium is desirable as the medium in this case, however, a medium that contains serum may also be used. Examples of media that have excellent characteristics for culturing mammalian cells like hybridomas, for example, include MEM, Ham's F-10 medium, Ham's F-12 medium, RPMI 1640 medium, and eRDF [enriched RPMI-1640, DME and Ham's F-12] medium, for example. The sugar chain of the glycoprotein can be changed by using a 0-50 % concentration of the optimal sugar

concentration used in these media. As indicated in the application examples, changes in the sugar chain in this invention are affected greatly by the sugar concentration. Accordingly, it is desirable to have the serum dialyzed and have the sugars removed when using a cell line for which serum is mandatory for growth. When using sugars other than glucose, a glycoprotein with changed sugar chains can be obtained by replacing all or part of glucose at an optimal concentration for cell growth with other sugars. Examples of sugars that display such an effect in particular include monosaccharides such as fructose, mannose, galactose, glucosamine, and ribose, for example. The use of galactose, glucosamine, and ribose in particular is desirable. Examples of polysaccharides include chitosan, dextran sulfate, alginic acid, heparin, chitin, and mannans, for example. In particular, chitosan, dextran sulfate, and alginic acid are desirable. Alginic acid may be a salt, but sodium alginate in particular is desirable.

[0007]

Any culture method can be used for culturing cells as long as it is a culturing method suitable for the cell. Examples include suspension culture in a tank, attached culture with a microcarrier and urethane materials, and culture in hollow fibers, for example. It may be cultured in a culture solution at a conventional glucose concentration that is suitable for that cell until the cell reaches a certain density, and the medium may be exchanged by one that has the type or the concentration of sugar changed after reaching the stage for producing the glycoprotein with the converted sugar chain. With polysaccharides, a polysaccharide solution may be added to a general culture solution at a concentration of 1-1000 μg/mL. The glycoprotein from the culture solution may be separated and purified by a general purification and collection method. However, the change in the sugar chain can be detected as the difference in the molecular weight in the glycoprotein, therefore a separation and purification method like SDS gel electrophoresis and gel filtration methods, for example, by the weight difference molecular amount is suitable. Application examples will be indicated below, and this invention will be explained in more detail.

[0008]

Application Example 1

A method for changing the sugar chain of an antibody of a glycoprotein that is produced by culturing an antibody-producing hybridoma will be explained in this application example. In particular, the glycoprotein that is obtained in this application example is an antibody that has a different affinity due to the difference in the sugar chain.

(1) Preparation of media with different sugar concentrations and compositions

Using ERDF medium (refer to the official report for Japanese Kokai Patent Application No. Hei 3[1991]-180175), which is a serum-free medium developed by Murakami et al., as the basic medium, media that have the glucose concentration of this medium adjusted to 0, 1, 2, 5, 10, 20, and 36 mM are prepared. The glucose (Glc) concentration of the ERDF medium is considered optimal for cell culture at 20 mM. Also, media that have the sugar in this ERDF medium replaced by fructose (Fru), mannose (Man), galactose (Gal), N-acetylglucosamine (GlcNAc), and ribose (Rib) are also prepared. Furthermore, fetal calf serum (FCS) dialyzed in phosphate-buffered physiological saline (PBS), and FCS (dialyzed FCS) that does not contain monosaccharides such as glucose, for example, is prepared, and it is added to each medium at a 5% concentration.

[0009]

(2) Production of glycoprotein

Human-human hybridoma C5TN is used as the glycoprotein producing cell. C5TN is a sub line of the hybridoma HB4C5 (Murakami et al., In Vitro Cell, Develop. Biol., 21:593-596, 1985), which is a cell line that secretes human IgM type antibody that has an affinity with carboxypeptidase (will be referred to as CPA below), double-strand DNA (will be DNA below), and Candida cytochrome C (will be CytC below) (Tachibana, H., et al., Biochem. Biophys. Res. Commun., vol. 189, 625-632, 1992). The L chain of the antibody that this hybridoma secretes (will be referred to as C5TN antibody below) is a λ chain, and the inventors of this invention have clarified that it has the sugar chain binding site of -Ser-Gly-Asn-Ser-Ser-Asn-Ile-Gly- in the CDR1 region of a variable region (VL domain). This cell line is allocated by the University of Kyushu, Department of Agriculture in Graduate School, Cell Engineering Lab. Roller cultures of C5TN, each with 5 mL medium at a density of $5x10^4$ cells/mL, are cultured at 37°C under an atmosphere of a 5% carbon dioxide gas for 24 h, and the cells are collected. A culture supernatant that is cultured for 48 h under the same conditions is also collected.

[0010]

(3) Separation of glycoproteins with different sugar chains

The culture supernatant for each culture medium is provided for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Each λ chain is confirmed by the Western blot method that uses an anti-λ antibody (Figures 1 and 2). Four types of L chains are detected, and they are named L1, L2, L3, and L4 from the side with a larger molecular weight that is judged by the position to which they migrated. Only L4 is synthesized in the presence of

tsunicamycin, which is the sugar chain synthesis inhibitor. It is assumed that L4 is an antibody that does not bind sugar chains. Table 1 shows the antibody production in each medium.

[0011]

Table 1

| ① 培地糖組成 | (mM) | ② L鎖のタイプ |
|---|---|---|
| Glc | 0 | L1, L3, L4 |
| Glc | 1 | L1, L3, L4 |
| Glc | 2 | L1, L3, L4 |
| Glc | 5 | L1, L2, L3, L4 |
| Glc | 10 | L1, L2 |
| Glc | 20 | L1, L2 |
| Glc | 36 | L1, L2 |
| Fru | 20 | L1, L2 |
| Man | 20 | L1, L2 |
| Gal | 20 | L1, L2, L4 |
| GlcNAc | 20 | L1, L3, L4 |
| Rib | 20 | L1, L3, L4 |

Key: 1 Sugar composition of the medium
2 Type of L chain

[0012]

An antibody with L2 that cannot be obtained under general culture conditions is obtained by the change in the concentration of glucose or sugar in the medium. This antibody is separated from the other antibodies by SDS-PAGE. This cell, as described above, produces an antibody that includes the λ chain (L chain) with 4 molecular weights, and the difference in the molecular weight in this λ chain (L chain) is due to the difference in the molecular weight of the sugar chain.

[0013]

Application Example 2

Change in antibody characteristics due to differences in sugar chains

The C5TN antibody has a sugar chain bound to L chain, and this sugar chain is converted to a sugar chain with different molecular weights as in (3) in this invention. The change in the antibody specificity due to this change in the sugar chain is confirmed in the manner below. The antigen specificity of the antibody can be measured by an enzyme-linked immunosorbent assay

method (ELISA) that uses any antibody-coated microplate (H. Murakami et al., Animal Cell Technology: Basic &; Applied Aspects, pp. 547-551, 1992, Kluwer Academic Publishers). A 200 ng/mL solution of the C4TN antibody cultured in each sugar is prepared, and the antigen specificity in this antibody solution is measured. The measured value is indicated by the percentage of the reactivity of the antibody cultured and obtained in 20 mM Glc as 100%. Figures 3 and 4 show the results. When the Glc content is changed, L3 and L4 increase, and the CPA affinity and the CyTC affinity also decrease, but the DNA affinity reaches the peak at 2 mM. When changing the sugar composition in the medium, the affinity of the antibody shows a completely different result from the antibodies cultured and obtained with Glc by the addition of Gal, GlcNAc, and Rib (Figures 3 and 4). It is thought to be a result of the change in the structure of the sugar chain that is bound to the antibody.

[0014]

Application Example 3

Using the ERDF basic serum-free medium that has the glucose concentration adjusted to 10 mM, the C5TN hybridoma cells are cultured under the same conditions as Application Example 1. More precisely, the C5TN hybridoma is roller-cultured at a density of $5 \times 10^4$ cells/mL for 6 h. Afterwards the cells are collected, transferred to a culture solution (ERDF) with chitosan (1 μg/mL), dextran sulfate (100 μg/mL), or alginic acid added (100 μg/mL), cultured for another 48 h, then the culture supernatant is collected and concentrated by dialysis. The same culture conditions are used as in Application Example 1, 37°C under a 5% $CO_2$ atmosphere. For a control, a culture with only ERDF is obtained. This concentrated solution is provided for SDS-PAGE electrophoresis under reducing conditions, and the L chains of the antibody are specifically detected using the anti-human λ L chain antibody with the Western blot method. Figure 5 shows the results. 28 kD and 30 kD L chains increased due to addition of each of the monosaccharides, compared to the culture with glucose only, which is a result that confirms the change in the sugar chain in the antibody. The antibody obtained is only the IgM type antibody in all of them.

[0015]

Application Example 4

The change in the antibody affinity in the antibody that is produced in Application Example 3 and has the sugar chain changed is confirmed by the method disclosed in Application Example 2. Using an antigen-coated microplate, the same measurement is obtained, and the reactivity of each antigen is measured by the ELISA method. The reactivity of each antigen is observed by the change in color by the ELISA method as the change in the light absorption at

405 nm. Figures 6-8 show the results. It has been confirmed that the reactivity of the antibody with the antigen has changed due to the changes in the sugar chain.

[0016]

Effect of the invention

The type and the molecular weight of the sugar chain that is added to a protein can be modified by this invention and a glycoprotein that has its characteristics changed can be produced by controlling the culture conditions, including the sugar composition or the sugar concentration, etc., of monossacharides in the medium, including ribose, galactose, and glucomisane, for example, upon glycoprotein production by culturing an animal cell. The type or the molecular weight of the sugar chain can also be modified and a glycoprotein with its characteristics changed can also be produced by adding polysaccharides such as alginic acid, dextran sulfate, and chitosan, for example, to the medium. Glycoproteins with different types or molecular weights of sugar chains can be obtained by this method. The bioactivity of the glycoprotein can also be changed by the modification of the sugar chains.

Brief description of the figures

Figure 1 shows the detection patterns of the λ type antibody that is obtained from C5TN cells cultured by changing the glucose concentration by the SDS-PAGE Western blot method in Application Example 1.

Figure 2 shows the detection patterns of the λ type antibody that is obtained from C5TN cells cultured by changing the glucose to another sugar by the SDS-PAGE Western blot method in Application Example 1.

Figure 3 shows the change in the antigen affinity of the antibody that is obtained from C5TN cells cultured by changing the glucose concentration in Application Example 2.

Figure 4 shows the antigen affinity of the antibody that is obtained from C5TN cells cultured by changing the glucose to another sugar in Application Example 2.

Figure 5 shows the detection patterns of the human λ type antibody that is obtained in Application Example 3 by the SDS-PAGE Western blot method.

Explanation of the symbols

Lane 1: Molecular size marker

Lane 2: Control

Lane 3: Chitosan added (1 μg/mL)

Lane 4: Dextran sulfate added (100 μg/mL)

Lane 5: Alginic acid added (100 μg/mL)

Figure 6 shows the change in the affinity of the antibody that is obtained in Application Example 3 for CPA.

Figure 7 shows the change in the affinity of the antibody that is obtained in Application Example 3 for CytC.

Figure 8 shows the change in the affinity of the antibody that is obtained in Application Example 3 for dsDNA.

Explanation of the symbols

-O-: Control

- □ -: Chitosan added (1 μg/mL)

-●-: Dextran sulfate added (100 μg/mL)

-Δ-: Alginic acid added (100 μg/mL)

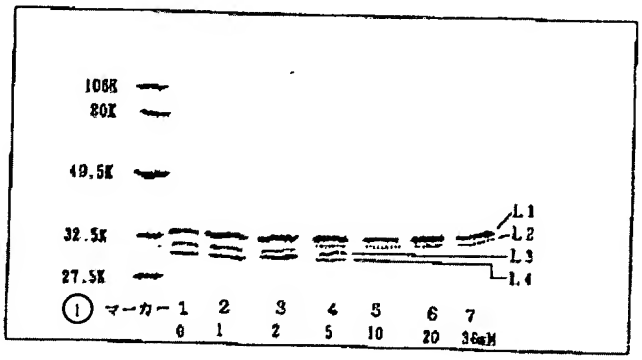


Figure 1

Key: 1 Marker

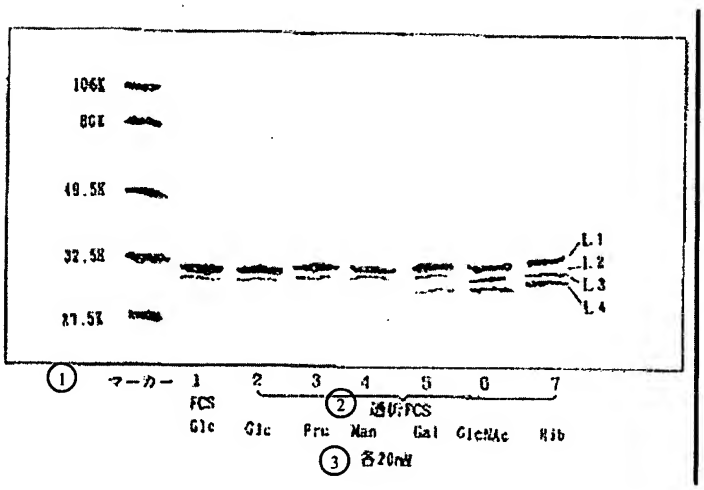


Figure 2

Key: 1 Marker
2 Dialyzed FCS
3 Each 20 mM

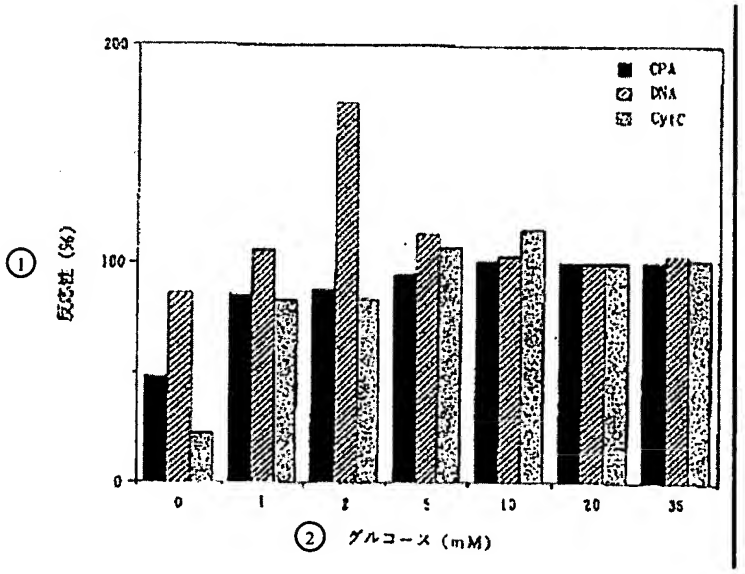


Figure 3

Key: 1 Reactivity (%)
2 Glucose (mM)

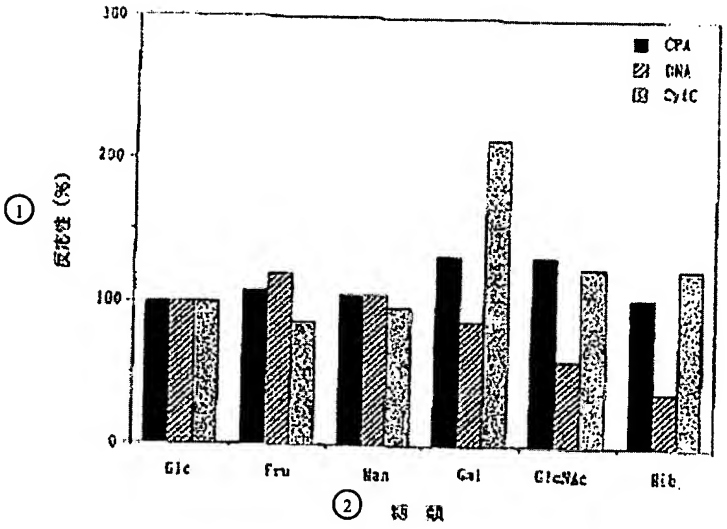


Figure 4

Key: 1 Reactivity (%)
2 Sugar chain

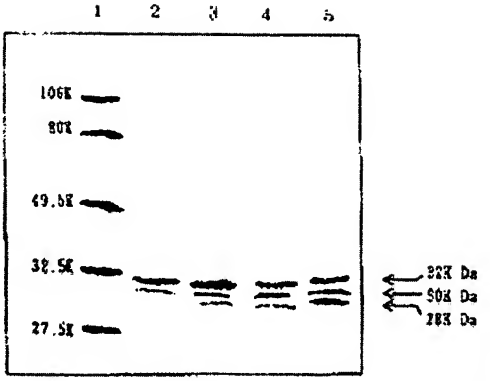


Figure 5

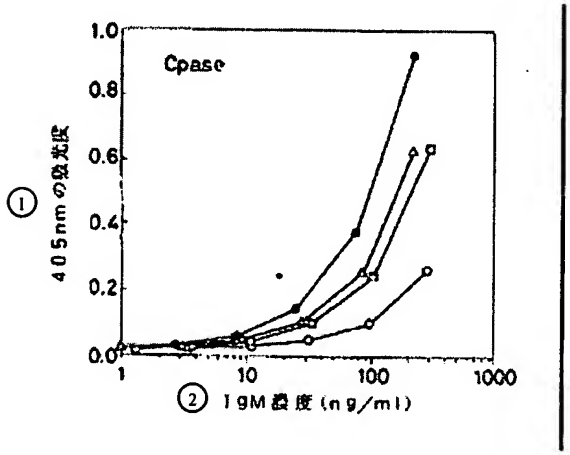


Figure 6

Key: 1 Absorption at 405 nm
2 IgM concentration (ng/mL)

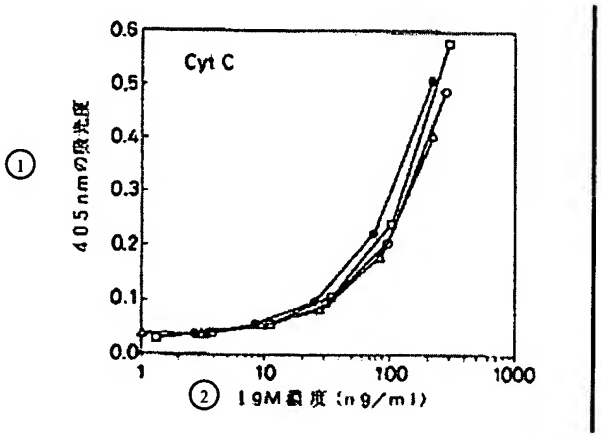


Figure 7

Key: 1 Absorption at 405 nm
2 IgM concentration (ng/mL)

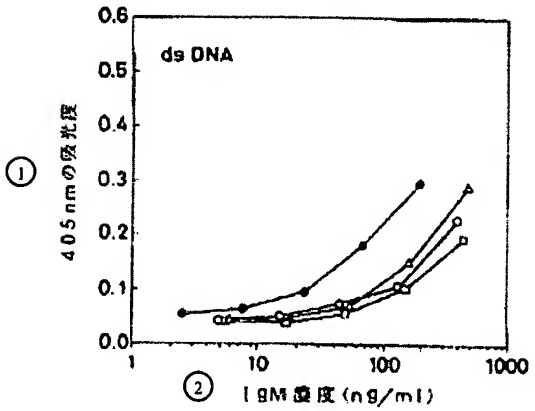


Figure 8

Key: 1 Absorption at 405 nm
2 IgM concentration (ng/mL)